INFLUENZA

III. Rapid Alterations in the Respiratory Rate of Embryonated Eggs Apparently Caused by Influenza Virus Toxin*

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Following the intra-allantoic injection of influenza A virus in embryonated eggs, a latent period of 4 to 6 hours has been observed, during which little or no active virus can be recovered from any of the embryonic fluids or tissues (1, 2).

Microbial toxins probably exert their effect by altering cellular metabolism. Pappenheimer and Hendee have suggested that the diphtheria toxin, for example, may be a cytochrome *b* which interferes competitively with mammalian cytochrome *b*, and thus inhibits intracellular respiration (3). It therefore seemed possible that the existence of an influenza virus toxin might be demonstrated, and its concentration measured, by studying the oxygen consumption of fertile eggs during the early part of the latent period of virus multiplication. The method employed somewhat parallels that used for the demonstration of rickettsial toxins (4–7) in connection with which the initial assumption was made that any deaths occurring in mice shortly after rickettsial injection would be ascribable to toxins rather than rickettsial multiplication.

The data to be presented here indicate that the allantoic fluid of fertile eggs infected with influenza virus contains a factor which when injected into a new series of eggs, profoundly modifies the respiration of the embryos. This toxic factor persists after infectivity has been destroyed by heating.

Material and Methods

The inoculum used consisted of pooled allantoic fluids from fertile eggs inoculated with the PR8 strain of influenza virus A, and collected after 36 hours of growth. This allantoic fluid had an infectivity titre of $10^{-8.5}$. It was injected without dilution into series of fertile eggs after 11 days of incubation at 37.5°C. In some experiments, amounts of infected fluid ranging from 0.125 to 3.0 cc. were injected; in other experiments, 1.0 cc. and 3.0 cc. of the fluid were injected after heating at 56°C. for periods of time ranging up to 60 minutes. Both the intra-allantoic and the yolk sac routes of introduction were used. Oxygen consumption determinations were made 90 minutes after the injection of the fluid. For controls, in addition

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to the heated infected fluid, normal allantoic fluid, both heated and unheated, and physiological salt solution were used. In the process of heating the infected fluids, the times at which infectivity and hemagglutinating ability disappeared were determined.

Methods for determining oxygen uptake have been described previously (8) and methods for titrating infectivity and hemagglutination are described in immediately preceding papers in this journal.

EXPERIMENTAL RESULTS

From the graph shown in Fig. 1, it can be seen that amounts of allantoic fluid up to and including 0.5 cc. injected intra-allantoically, had no significant effect on the oxygen consumption of the receptor eggs. When the amount

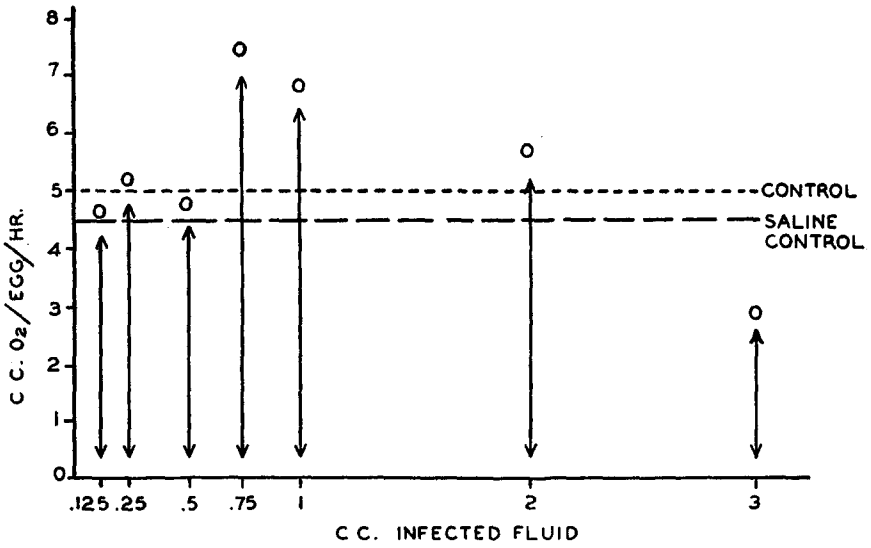

FIG. 1. Effects on oxygen consumption of embryonated eggs resulting from the injection of different amounts of infected allantoic fluid. All determinations were made approximately 90 minutes after intra-allantoic injection of the fluid.

of fluid was increased to 0.75 cc., however, a marked increase in oxygen consumption ($2\frac{1}{2}$ times that necessary to be regarded as significant (8)) was produced. An increase is still seen with 2.0 cc. of fluid, but the eggs receiving 3.0 cc. showed a significant drop in oxygen uptake. No significant increase was noted in the eggs receiving equivalent amounts of saline solution.

Injection of the infected fluid into the yolk sacs of eggs caused similar alterations in respiration. No significant alterations in respiration were caused by the intra-allantoic injection of equivalent amounts of heated or unheated normal allantoic fluids.

From a study of Fig. 2, it is apparent that the stimulating effect of 1.0 cc.

of infected fluid is not destroyed by heating for 20 minutes, but disappears after heating for 30 minutes. In terms of the data shown in Fig. 1, this should indicate that the hypothetical toxin is 50 per cent destroyed in 30 minutes at this temperature. When 3.0 cc. of fluid was injected, the depressing effect

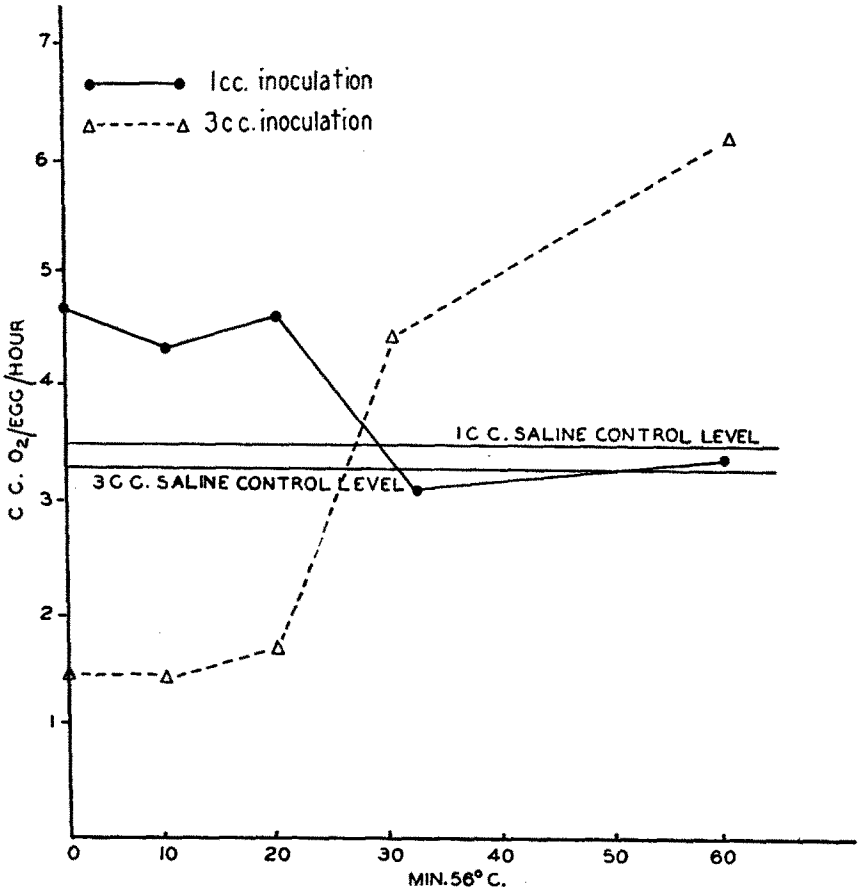

FIG. 2. Effects on oxygen consumption of embryonated eggs resulting from the intra-allantoic injection of 1 cc. and 3 cc. of infected allantoic fluid after heating for various time periods.

on respiration was changed to a stimulating effect at some time between 20 and 30 minutes of heating at 56°C. Again this agrees roughly with the data in Fig. 1, if we assume 50 per cent destruction of the toxin. At the end of 60 minutes, the peak of stimulation of respiration was reached, which would indicate about 75 per cent destruction.

It should be noted that the solid line in Fig. 2 is essentially the reverse picture of the first part of the data seen in Fig. 1, and that the dotted line in Fig. 2 is the reverse of the remainder of the data seen in Fig. 1. In one instance, increasing concentrations of toxin were introduced by increasing the amounts of the fluid injected, while ir the second experimen decreasing concentrations of toxin were introduced because of prolongation of the period of heating.

Infectivity of the fluid was completely lost by heating for 20 minutes. Hemagglutination disappeared after heating the fluid for 10 minutes.

DISCUSSION

Although the production of toxins by rickettsiae is well established and has been rather minutely studied (4–7) little information is available concerning viral toxins.

Rake and Jones (9) have shown the probable formation of a toxin by the virus of lymphogranuloma venereum. Using 36 million infective units, these workers were able to produce early death with hepatic necrosis in mice.

Henle and Henle (10–13) and Hale and McKee (14) have described tonic and clonic convulsions in mice inoculated intracerebrally with concentrated preparations of allantoic fluid infected with influenza virus which they believed to be caused by a toxin. These mice die 24 to 72 hours following inoculation. Those workers have also reported that the toxic factor cannot be separated from the virus particle. In eggs, however, they were able to show that the infectivity titre often reached a peak 24 hours following the inoculation of the seed virus, whereas the maximum toxicity of the allantoic fluid occurred 48 hours after inoculation. Heat, formalin, and irradiation with ultraviolet light were found to cause more alteration in infectivity than in toxicity.

Parodi *et al.* (15) attributed the depression of respiration in infected eggs to interference with gaseous exchange as a result of thickening of the chorioallantoic membrane.

The experimental results reported here indicate a direct effect of the toxin on cellular metabolism, particularly in view of the fact that the effect was obtained when the infected fluid was injected by the yolk sac route.

Our results suggest that the influenza virus toxin differs from rickettsial toxins and from the toxin produced by the lymphogranuloma venereum virus. These latter toxins are labile, and are closely associated with active rickettsiae or virus particles, while the influenza virus toxin which we have studied may be separated from the active virus particles by heating. Thus the influenza toxin appears more analogous to bacterial toxins than to rickettsial toxins and lymphogranuloma venereum toxin. Whether the influenza toxin will prove to be antitoxinogenic remains to be seen. Attempts are being made to concentrate and purify this toxin, as a preliminary to further studies of its nature and activity.

The method described here may prove of value in the study of the physiological action of other types of toxins, since it is sensitive, rapid, and efficient as compared to the use of animals.

SUMMARY

Allantoic fluid from embryonated eggs infected with influenza A virus contains a toxic agent which can be demonstrated and quantitatively measured by its rapid effect on oxygen consumption when it is introduced in new series of fertile eggs.

The effects were measured 90 minutes after the injection of the infected fluid, and were seen following both intra-allantoic injection and injection into the yolk sac.

This toxin, in concentrations resulting from the injection of 0.5 cc. or less of the infected fluid, has no effect on oxygen consumption. The injection of 0.75 to 2.0 cc. of the fluid strikingly increases the oxygen consumption of the fertile eggs, while the injection of 3.0 cc. markedly depresses respiration. A similar reversal and eventual loss of the effect of the toxin on respiration were noted when the concentration of toxin was progressively diminished by heat inactivation.

The toxic agent is slowly inactivated by heating at 56°C., but is effective long after infectivity and hemagglutinating ability have been destroyed. In this respect the agent differs from rickettsial and lymphogranuloma venereum virus toxins.

The method described may be of value in studying the physiological effects of other toxic agents.

BIBLIOGRAPHY

1. Henle, W., Henle, G., and Rosenberg, E. B., *J. Exp. Med.*, 1947, **86,** 423.
2. Hoyle, L., *Brit. J. Exp. Path.*, 1948, **29,** 390.
3. Pappenheimer, A. M., and Hendee, E. D., *J. Biol. Chem.*, 1947, **171,** 701.
4. Gildemeister, E., and Haagen, E., *Deutsch. med. woch.*, 1940, **66,** 878.
5. Otto, R., and Bickhardt, R., *Z. Hyg. u. Infektionskrankh.*, 1941, **123,** 447.
6. Bengston, I. A., Topping, N. H., and Henderson, R. G., *Nat. Inst. Health Bull., U.S.P.H.S., No. 183,* 1945, 25.
7. Smadel, J. E., Jackson, E. B., Bennett, B. L., and Rights, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62,** 138.
8. Greiff, D., and Pinkerton, H., *J. Exp. Med.*, 1948, **87,** 175.
9. Rake, G., and Jones, H. P., *J. Exp. Med.*, 1944, **79,** 463.
10. Henle, W., and Henle, G., *Science*, 1944, **100,** 410.
11. Henle, W., and Henle, G., *Am. J. Med. Sc.*, 1945, **210,** 362.
12. Henle, W., Henle, G., Stokes, J., and Maris, E. P., *J. Immunol.*, 1946, **46,** 163.
13. Henle, W., and Henle, G., *J. Exp. Med.*, 1946, **84,** 639.
14. Hale, W. M., and McKee, A. P., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59,** 81.
15. Parodi, A. S., Lajmanovich, S., Pennimpede, F., and Mittelman, N., *J. Immunol.*, 1948, **58,** 109.